MycoSM Mycology Short Master:
LE INFEZIONI FUNGINE: UN PROBLEMA EMERGENTE DI SANITA' PUBBLICA, DALLA EZIOLOGIA ALLA TERAPIA

Le micosi profonde opportunistiche: Criptococcosi e Pneumocistosi

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Cryptococcosis

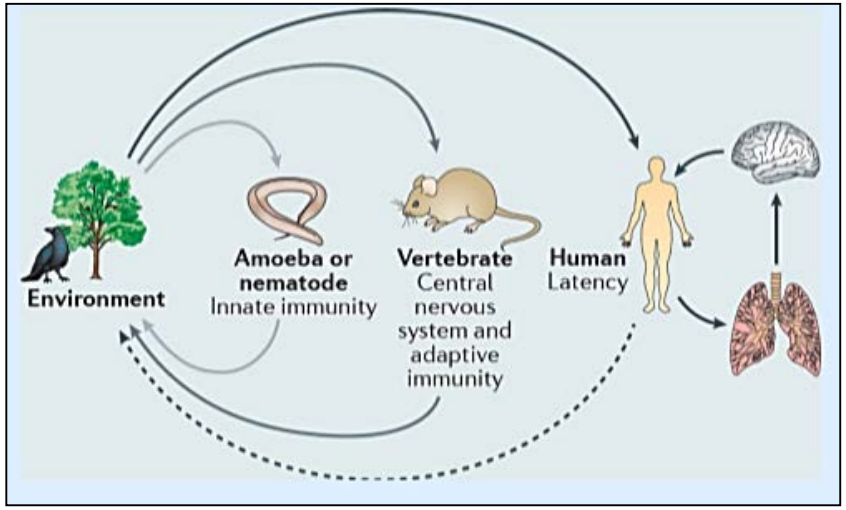
- Fungal infection of people and animals
- Acquired by the inhalation of air-borne organisms or accidental cutaneous injection from the environmental
- Cutaneous, pulmonary, central nervous system form and disseminated – immunosuppression is important
- NOT A ZOONOSIS
- NOT A CONTAGIOUS DISEASE
- Animals as sentinels: occasional outbreaks (animals and humans) from exposure to common environment source

The evolution of virulence in Cryptococcus

Cryptococcal pathogenesis **is not from direct selection for virulence within a mammalian host** but rather by the evolution of traits in response to other **selective pressures in <u>both environmental and animal</u>**

Polysaccharide capsule
Laccase activity
Synthesize melanin

Desiccation, ultraviolet light ect



Selective pressures of reptilian, avian or mammalian hosts

Ability to perturb adaptive immunity, preventing complete fungal clearance and resulting in **latent infections**

Survival and replication in **free-living phagocytic amoebae (predators)**

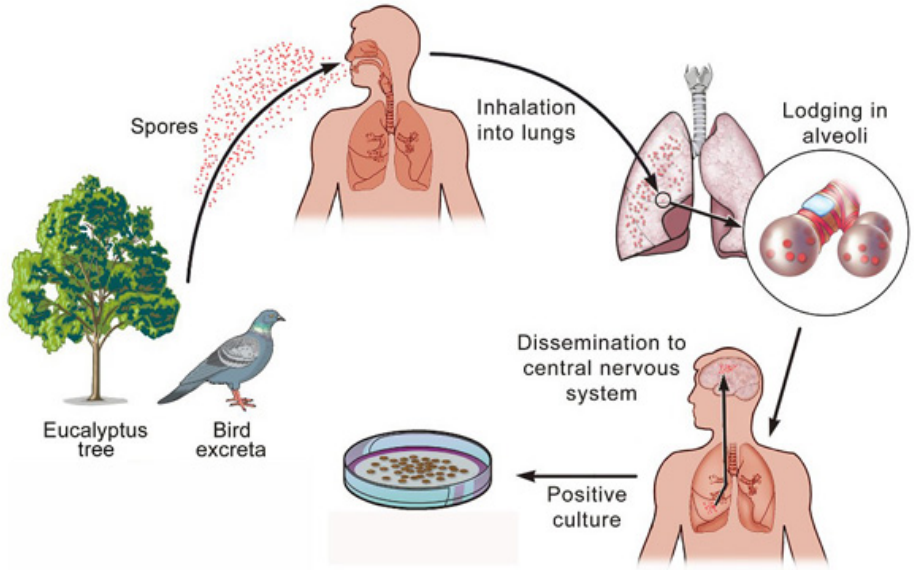
Survival within phagocytic white blood

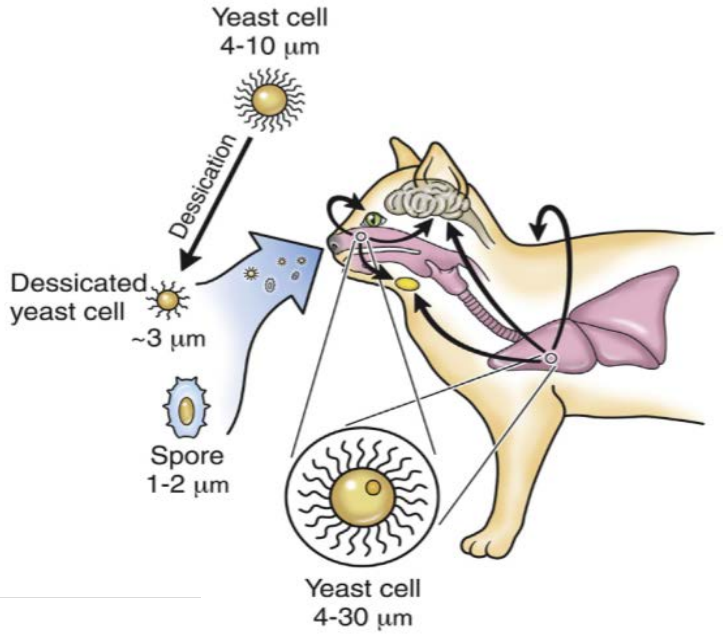
Pathogenesis parallels with humans

Lungs in humans

Primary site of infection

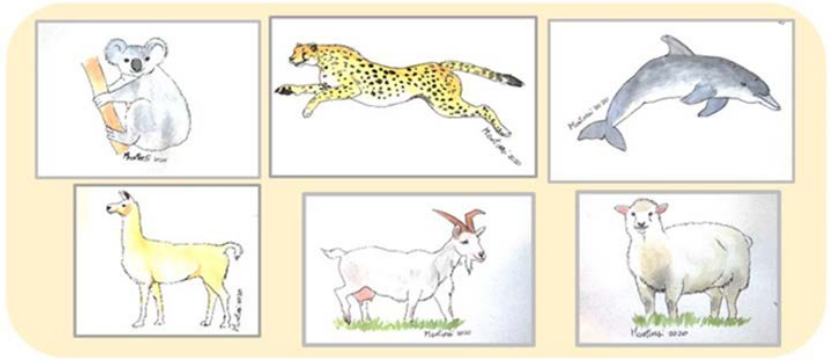
Nasal cavity in cats, dogs, koalas, and psittacine birds



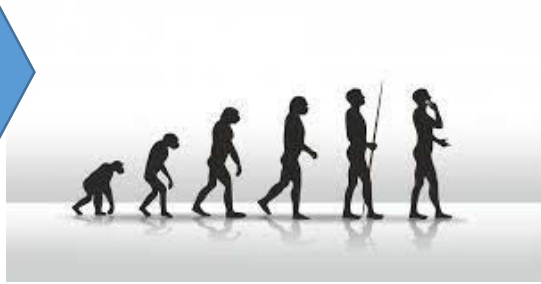


- ❑ Size of inoculum
- ❑ Virulence of cryptococcus strains
- ❑ Host immunity

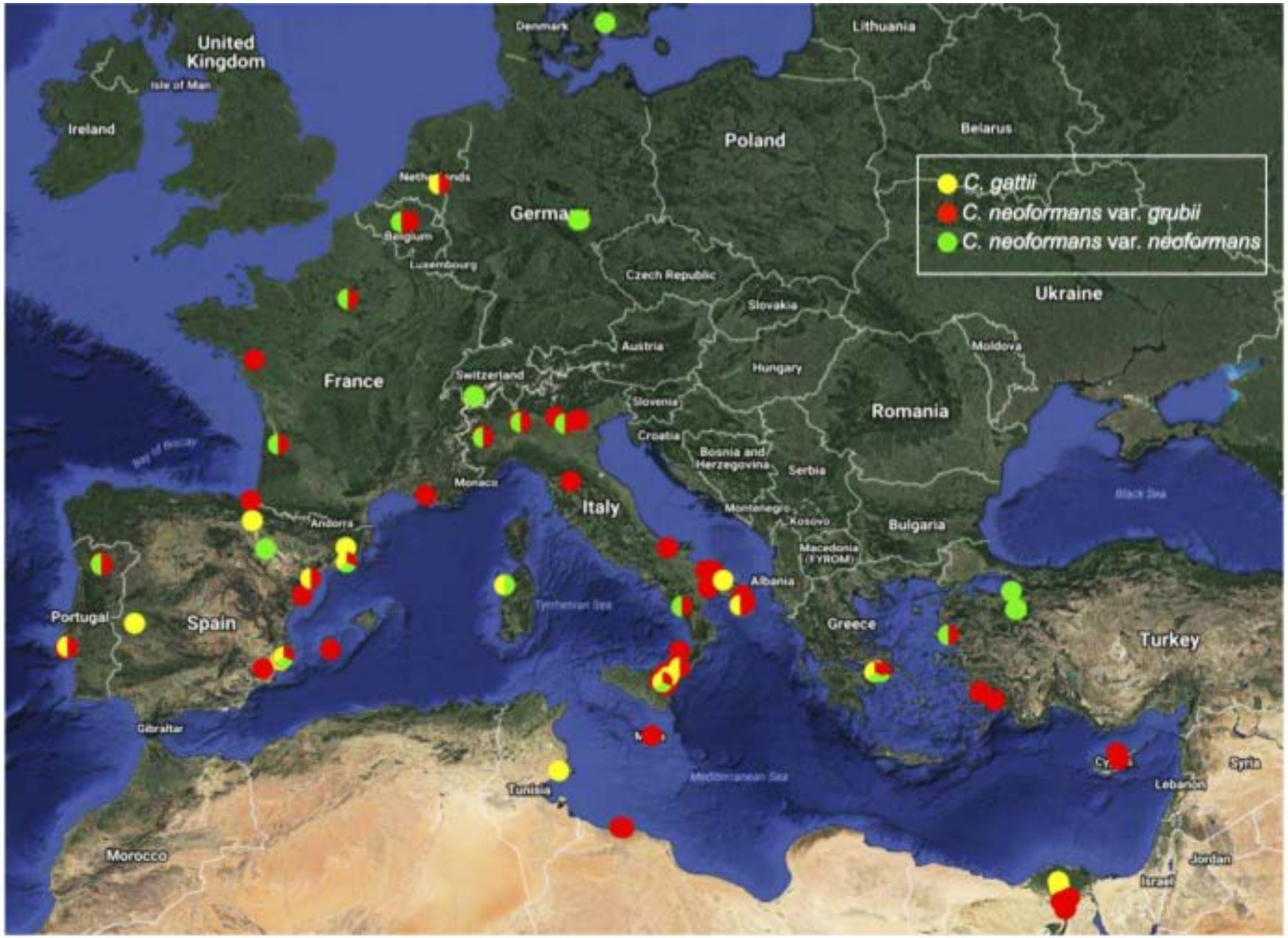
Cryptococcosis clinical signs in hosts



- Asymptomatic colonization
- Cryptococcal rhinitis
- Respiratory distress (lungs and air sacs)
- Central nervous system (CNS) involvment - meningoencephalitis
- Ocular signs (midriasis, blindness)
- Localised cutaneous lesions
- Systemic forms



Cryptococcus distribution in Europe based on envirnmental and animal isolates



From Cogliati *et al*., 2017. Environmental Microbiology 19(10), 4318–4325

1. Asymptomatic colonization

- Presence/isolation (positive culture) of the organism from a surface anatomical site without clinical signs and/or infection (by definition a negative IMMY or CrAG test)

- Asymptomatic colonisation of the respiratory tract is more common than clinical disease (cats)

Asymptomatic Cn/Cg carriage					
	Dog	(%)	Cat	(%)	
★ Australia	8/54	14	3/45	7	Malik *et al* ., 1997
★ British Columbia	4/280	1,1	3/94	4,3	Ducan *et al* ., 2005
Italy	nd	nd	12/766	1,6	Danesi *et al* ., 2014

★ **Endemic area**

2. Cryptococcal rhinitis

Nasal form:

- presenting as a **chronic sinonasal disease**, either alone or together with local spread to the skin, subcutis, bones and regional (submandibular) lymph nodes
- sneezing, epistaxis (bleeding) and nasal discharge
- granulomatous protuberances occasionally at the nares
- destruction of adjacent facial bones

2. Cryptococcal rhinitis

Cryptococcal rhinitis -
Cryptococcus gattii - FIV+ cat

Facial distortion: suggestive of both rhinusinusitis and neoplasia

Pennisi *et al*., 2013. Journal of Feline Medicine and Surgey 15, 611-618

2. Cryptococcal rhinitis

Nasal swelling caused by cryptococcosis in a 6-year- old female domestic long-hair cat.

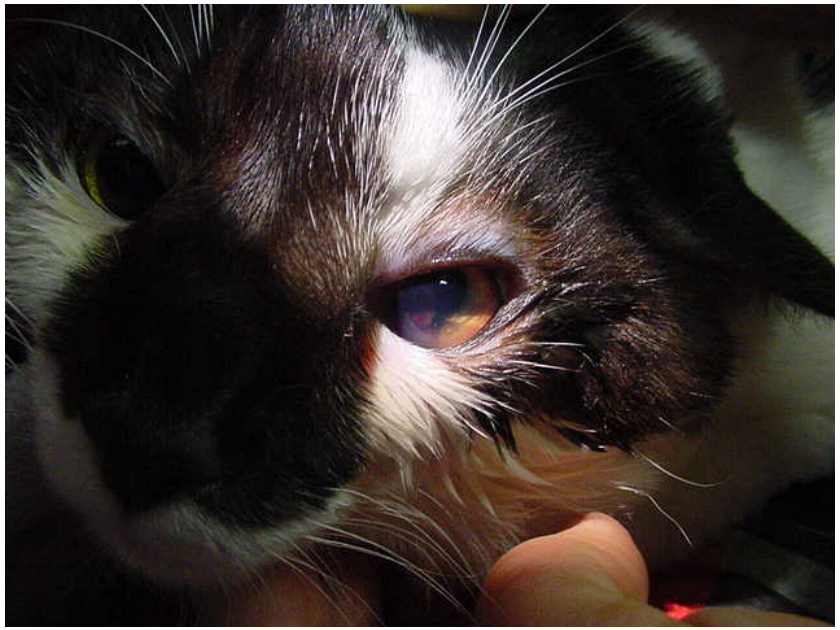
FeLV/FIV-negative 2.5-year-old Ragdoll Queen with bilateral mandibular lymphadenomegaly. Biopsy of mandibular lymph nodes confirmed pyogranulomatous lymphadenitis secondary to *Cryptococcus neoformans* infection

http://vetbook.org/wiki/cat/index.php?title=Cryptococcus_spp

3. Central nervous system

- **Infection may extend through**
  - **cribriform plate into the olfactory bulbs and olfactory tract**, giving rise to meningoencephalitis
  - **concurrent cryptococcal optic neuritis and secondary retinitis -** anatomic proximity of the optic nerves

Ocular cryptococcosis



Cryptococcal disease – kerato-uveitis and cryptococcoma in the anterior chamber.

Bilateral mydriasis in a 8 year old cat with cryptococcal meningoencephalitis with optic neuritis and retinitis

Pennisi *et al*., 2013.

Cutaneous Cryptococcosis

- **Secondary involvement** from the nasal cavity infection (more common)

- **Localized cutaneous** can develop after inoculation of propagules after a cat scratch (rare)

- **Multifocal skin lesions** are the result of hematogenous dissemination and consist of papules and nodules

3. Systemic cryptococcosis

- Disseminated disease are more common in dogs than cats

- Gastro-intestinal tract involvement
- Pancreas
- Mesenteric lymph nodes
- Kidneys
- Myocardium
- Thyroid gland
- Eye
- Brain

Cryptococcosis in cats *vs* dogs

	Cats	Dogs
❑ **Asymptomatic colonization**	★★★	★★★
❑ **Cryptococcal rhinitis** (but not all)	★★★	★
❑ **Central nervous system (CNS) involvment -** meningoencephalitis	★★	★★★
❑ **Systemic forms**	★	★★★
❑ **Localised cutaneous lesions**	★★★	★

Clinical features

- ✓ Often a **chronic infection** (check if underlying diseases)
- ✓ **Upper respiratory tract nasal cavity more common**
- ✓ mandibular lymphoadenopathy
- ✓ single/multi-focal ulcerated or not **cutaneous mass**
- ✓ **Neurological signs**: depends on the location of lesions
- ✓ **Optic neuritis** and chorioretinitis
- ✓ Peripherical lymphoadenomegaly
- ✓ **Lameness** (osteomyelitis/arthritis)
- ✓ **Swollen digits**

- ✓ **Severe disseminated disease** (50%)
- ✓ nasal cavity, skin (less common than cats), lungs, lymph nodes, kidney, eye and CNS.
- ✓ **Rhinosinusitis** may be subclinical or mild
- ✓ Primarily signs of **gastrointestinal or pancreatic** involvement – vomiting, diarrhoea, abdominal pain
- ✓ **Cutaneous** involvment – uncommon – but as in cats, may be a marker for disseminated disease

Treatment and prognosis

“prognosis for many cats and dogs with cryptococcosis is good or excellent if they have diligent, co-operative owners who are prepared to medicate their pets for many months and pay for the costs of drugs and monitoring.”

- ❑ High cost therapy
- ❑ Requirement for multiple hospital visits
- ❑ Regular medication for protracted period
- ❑ Immunosuppressed animals – likely for persistent or progressive infection
- ❑ Surgical debulking for large cryptococcomas – before starting treatment can be helpful

Treatment

- AMB + Flucytosine – optimal therapy - with CNS cryptococcosis in cats (NOT FOR DOGS)
- Dogs often develop epidermal necrolysis (typically 10-14 day after starting therapy)
- FCY useful, effective, and moderately expensive drug. Rapid resistance might be develop when used alone. Used to improve the efficacy of other antifungal drugs
- Localised cutaneous disease – Azole monotherapy with Fluconazole is drug of choice (good penetration brain, eye, urinary tract, low cost, minimal adverse effects)
- To be continued – complete resolution of lesion and decrease on antigen titers
- Monitoring hepatoxicity - liver enzymes monthly

Treatment

- AMB is the most effective anticryptococcal agent - fungicidal - ability to permanently eradicate CNS infections
- The combination of AMB and flucytosine (FCY) - optimal therapy for cats - severe or widely disseminated disease - especially CNS involved
- Administration AMB IV or as an SC infusion if using Fungizone
- Nephrotoxic – largely reversible
- Newer forms (liposomal and lipid complex preparations) are not more effective – but less nephrotoxic – and more expensive

Cryptococcosis diagnosis

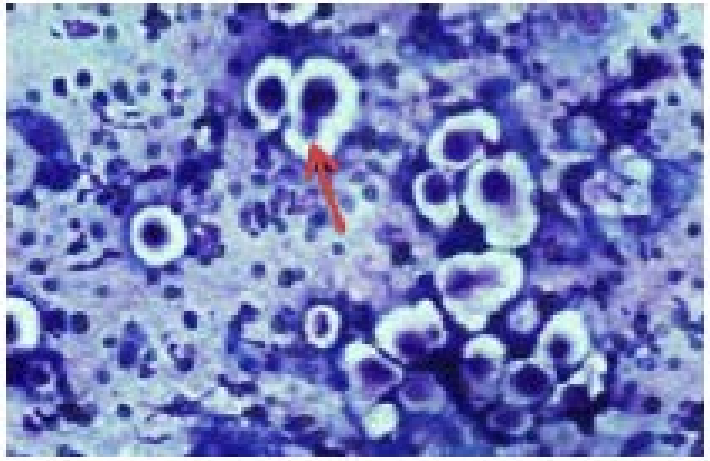
Evaluation of *Cryptococcus* in representative tissue specimens

- cytology
- culture
- histopathology
- Serology
- molecular methods ID – Fingerprintng, PCR, Real-time, ect

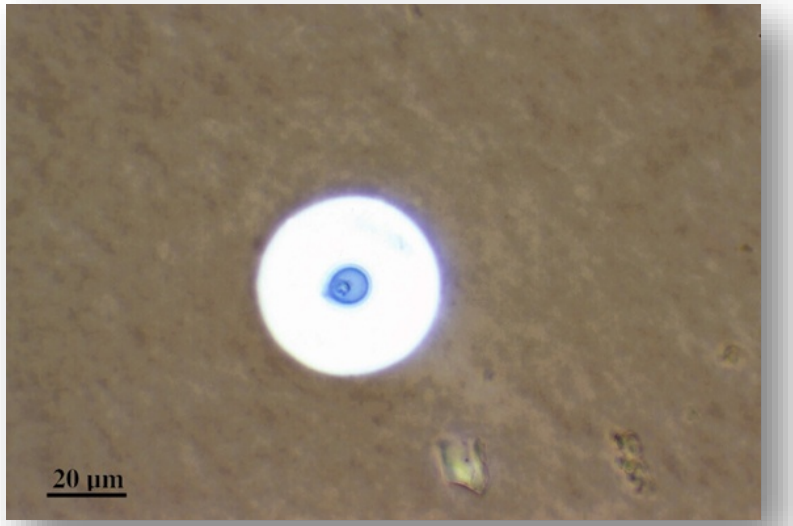
Suitable specimens

- ❑ Nasal swab/nasal washing
- ❑ Needle aspirates from mass lesions or enlarged lymph nodes
- ❑ Bronchoalveolar lavage specimes
- ❑ Pleural fluid
- ❑ CSF
- ❑ Urine

Cytology



Diff Quick stained smear of nasal exudate from cat with *C. neoformans* infection.
Note the prominent capsule and the narrow-necked budding (arrow).

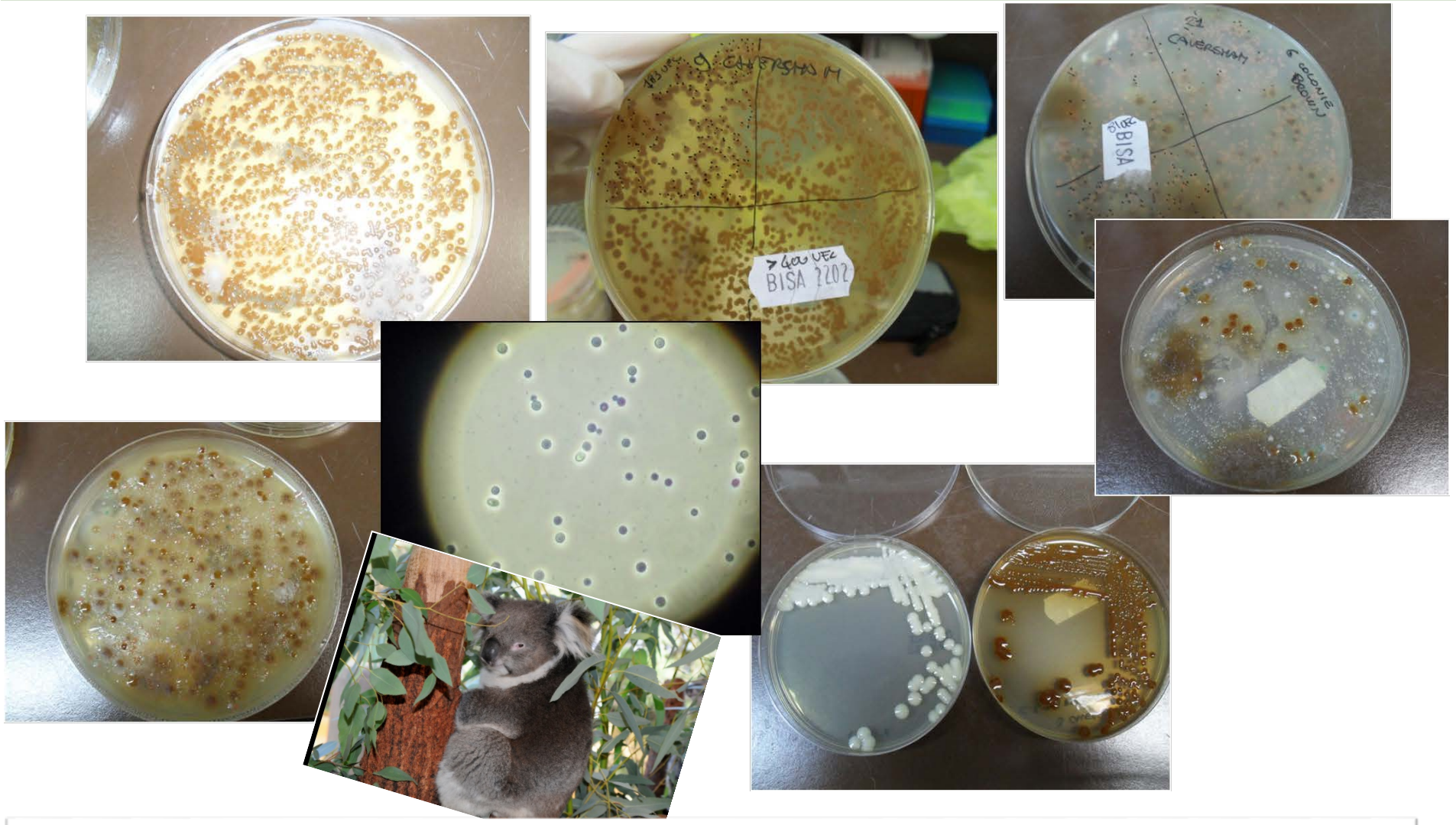


India ink preparation of cerebrospinal fluid from a patient with cryptococcal meningitis: cryptococcal yeast - *C. neoformans* - appears unstained and silhouette against a black background.

Fungal Culture

- Culture the organism before treatment is initiated
- Routine fungal media at 25/37°C
- Colonies visible 2/3 up to 10 days
- Isolation confirms the diagnosis
- **If isolated from a contaminated site (nasal cavity) – cytology or hystology support diagnosis of active infection**
- Yeast form is less likely to represent a laboratory hazard
- Subclinical colonisation occurs in some animals

Brown *Cryptococcus* colonies on niger seed agar



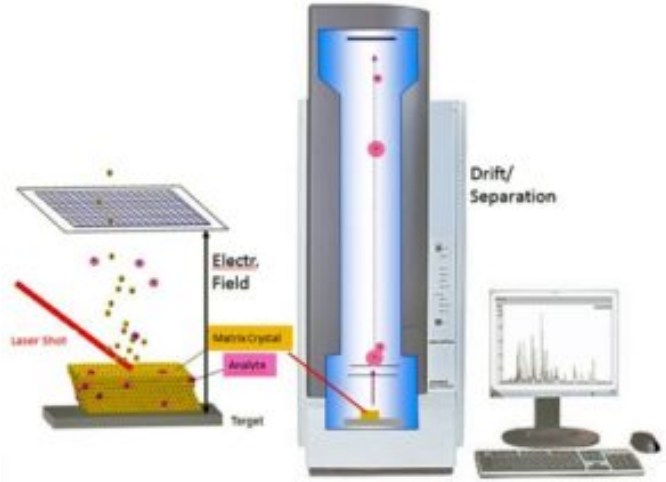
Characterisation of *Cryptococcus* strains isolated from mammals living in an environmental site with high cryptococcal presence in South-Western Australia

Patrizia Danesi,[1] Richard Malik,[2] Mark B. Krockenberger[2] and Wieland Meyer[2]

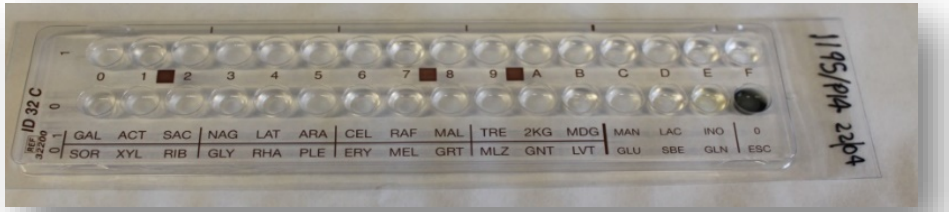
9th International Conference on Cryptococcus and Cryptococcosis (ICCC-9) in Amsterdam, 15–19 May 2014

Fungal ID from culture *Cn* vs *Cg*

MALDI-TOF MS
Matrix assisted laser desorption/ionization - time of flight mass spectrometry -



Biochemical tests

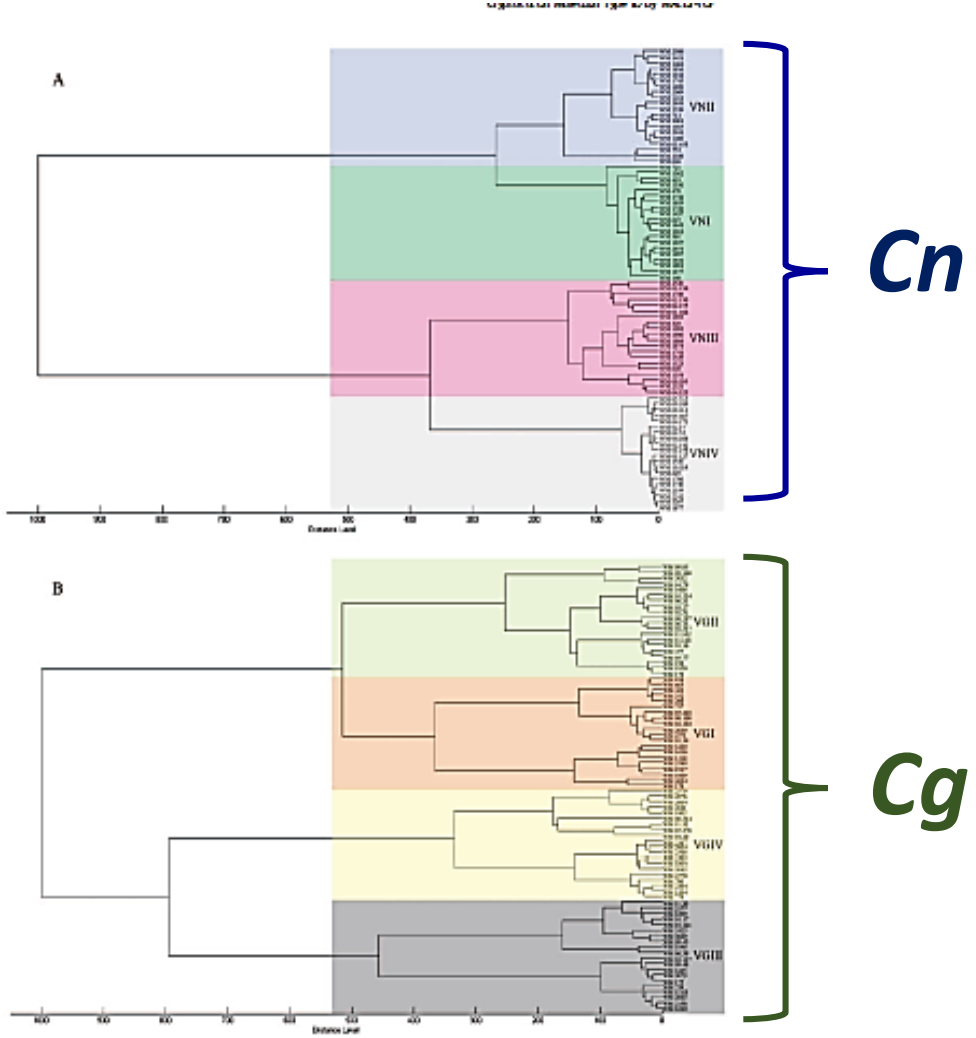


❑ **Molecular ID:**

❑ URA5 – RFLP (molecular type)
❑ PCR and sequencing
❑ MLST – molecular epidemiology

MALDI-TOF MS:

Major Molecular Types within *the Cn/Cg* species complex



Cn

Cg

Firacative C, Trilles L, Meyer W (2012).
MALDI-TOF MS Enables the Rapid Identification of the Major Molecular Types within the Cryptococcus neoformans/C. gattii species Complex
PLoS ONE 7(5): e37566. doi: 10.1371/journal.pone.0037566

Maldi-Tof: Identification *Cryptococcus neoformans/gattii* species complex

- Identification power - **limited by the robustness of the reference library used**
- **Supplementary library has successfully increased the performance for *non-neoformans Cryptococcus* spp. identification**
- Supplementary libraries entries are relatively **easy to generate**
- The possibility to create and to transfer an in-house library adds to the advantages of MALDI-TOF MS as an important tool for a rapid and cheap identification of pathogenic and saprophytic fungi

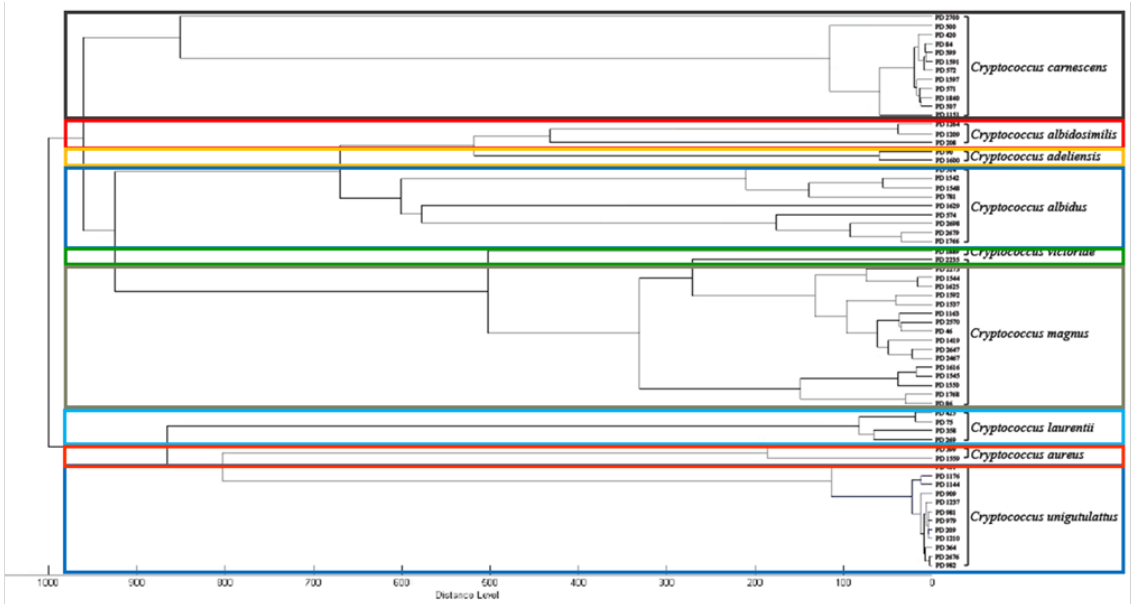


Figure 1. MSP dendrogram grouping *Cryptococcus adeliensis, C. albidosimilis, C. albidus, C.aureus, C. carnescens, C. laurentii, C. magnus, C. uniguttulatus*, and *C. victoriae* isolates according to the species level.

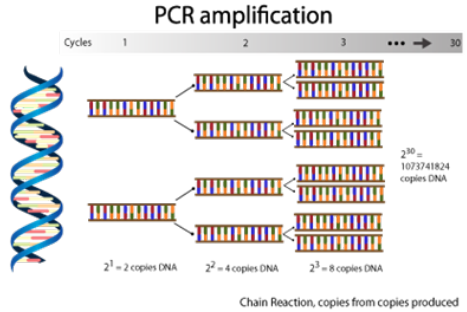
Med Mycol. 2014 Aug;52(6):659-66. doi: 10.1093/mmy/myu031. Epub 2014 Jun 20.

MALDI-TOF MS for the identification of veterinary non-C. neoformans-C. gattii Cryptococcus spp. isolates from Italy.

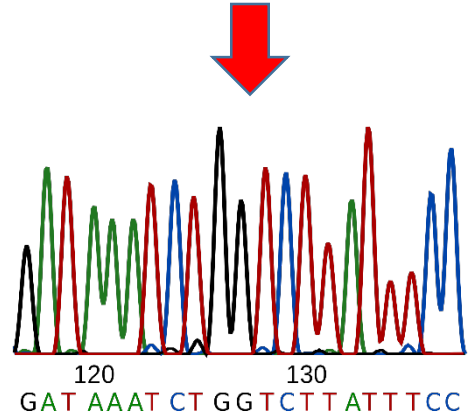
Danesi P[1], Drigo I[2], Iatta R[3], Firacative C[4], Capelli G[2], Cafarchia C[3], Meyer W[5].

PCR and sequencing

- Culture
- Tissue
- FFPE

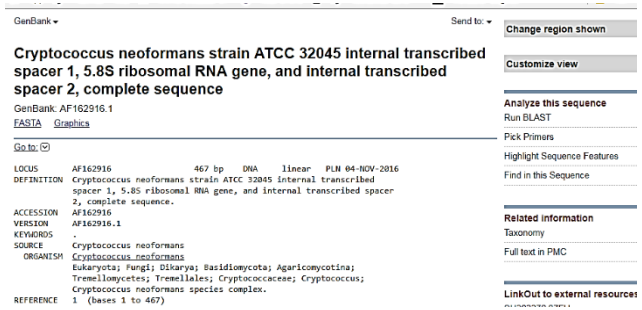


Universal primers ITS1/4



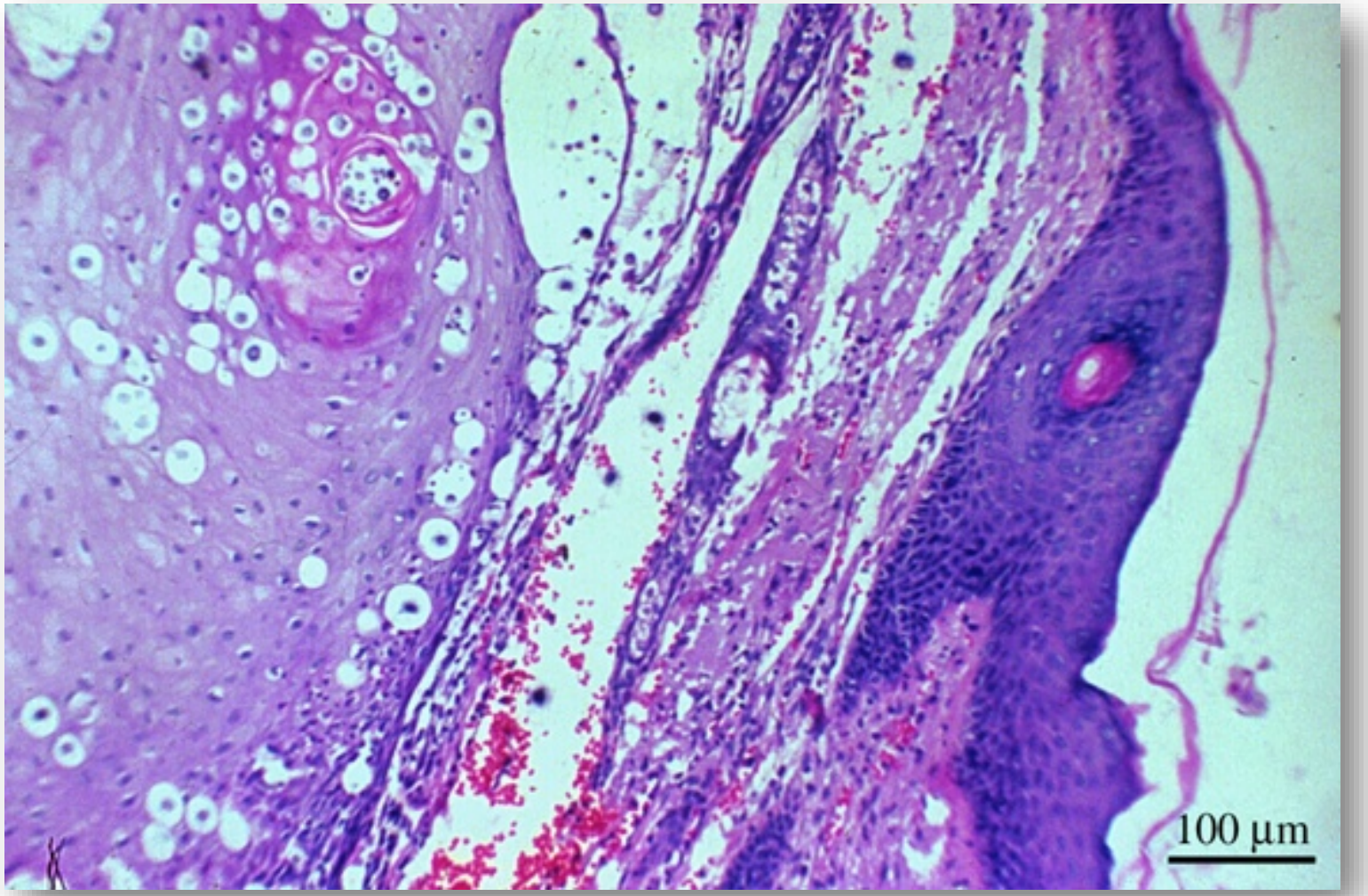
https://its.mycologylab.org/

https://wi.knaw.nl/Pairwise_alignment



In literature - large availability of primer set and PCR protocols according your aim and facility

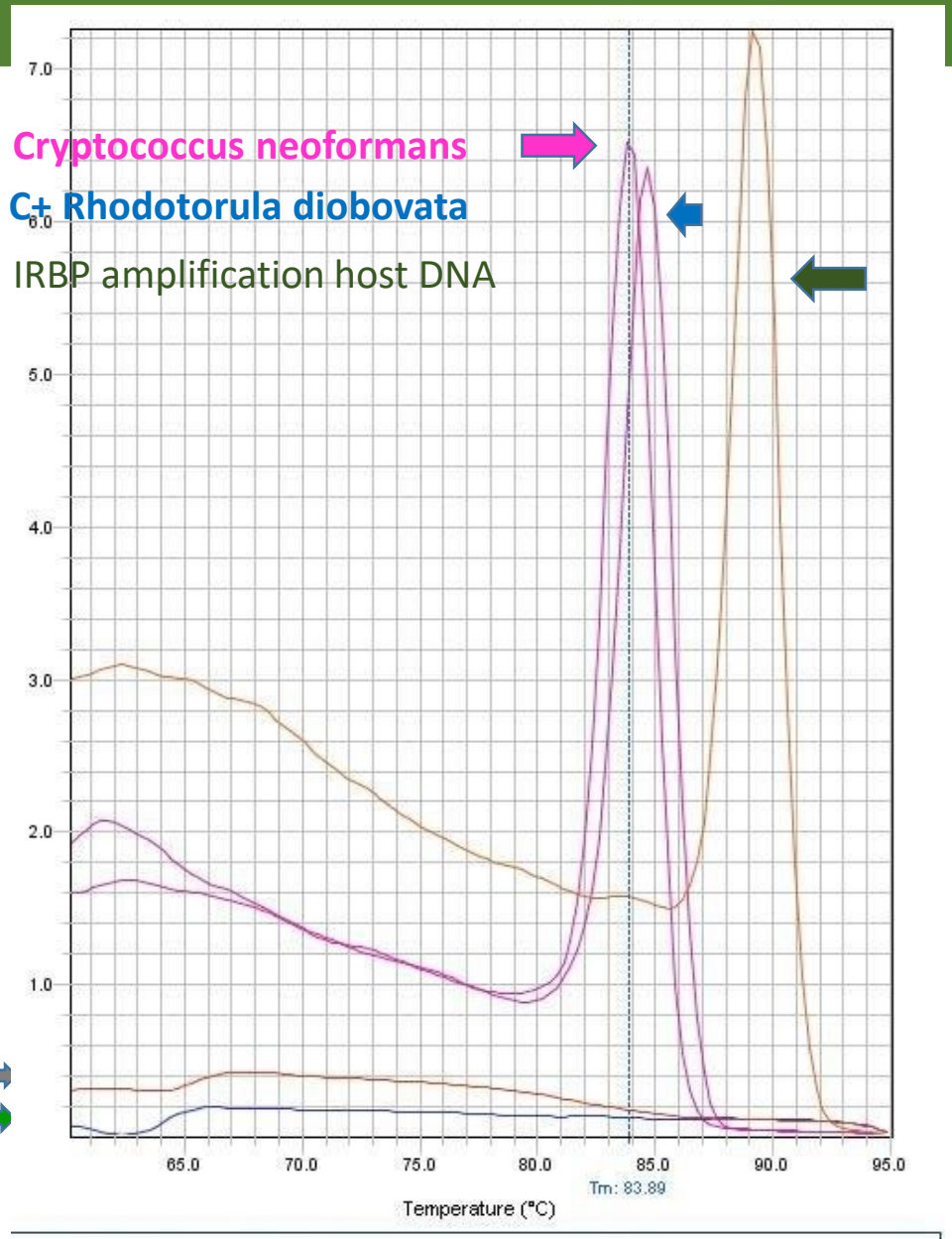
Histology: Hematoxylin-Eosin



Tissue section stained by Haematoxylin and eosin (H&E) showing numerous encapsulated yeast cells surrounded by a clear halo – the unstained capsule. *C. neoformans* was isolated. (Courtesy Dr G. Hunter, Adelaide, S.A.).

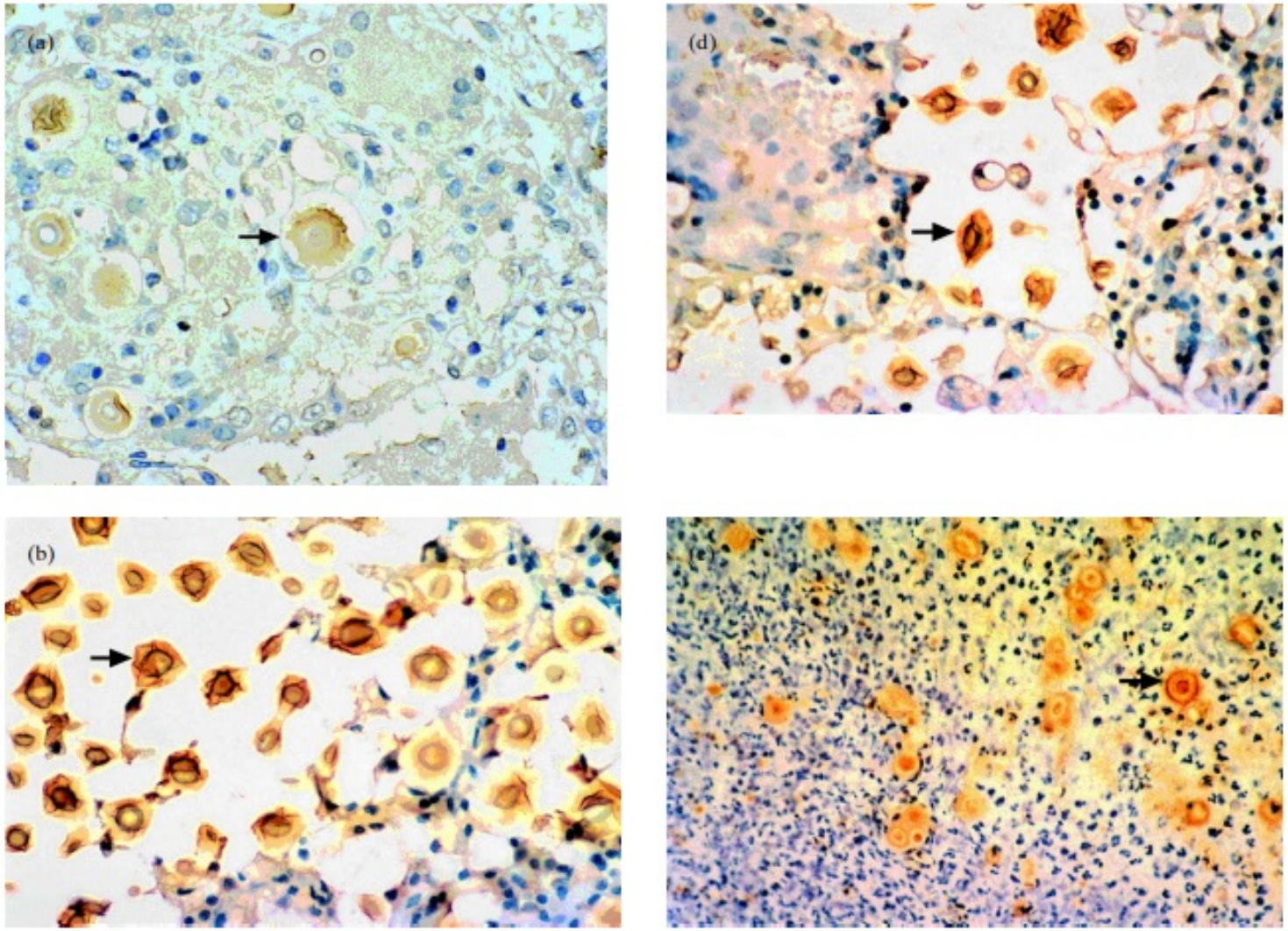
Molecular ID from Formalin- fixed paraffin- embebbed tissue (FFPE)

- Realtime PCR plus sequencing
- In-house designed primers targeting a portion of 26S LSU rRNA (210 – 230 bp) – good results from molecular identification from FFPE tissue



NPC – Negative process control
NTC – Negative template control

Immunohistochemistry



Krockenberger *et al*., 2001

Molecular epidemiology: MLST – multi-locus sequence typing

Consensus multi-locus sequence typing scheme for **Cryptococcus** neoformans and **Cryptococcus** gattii.

Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, Fisher M, Gilgado F, Hagen F, Kaocharoen S, Litvintseva AP, Mitchell TG, Simwami SP, Trilles L, Viviani MA, Kwon-Chung J.

Med Mycol. 2009;47(6):561-70. doi: 10.1080/13693780902953886. Review.

PMID: 19462334 Free PMC Article

In 2007, the ISHAM *Cryptococcus* working group member established to adopt MLST as the **gold standard technique for *Cn* and *Cg* molecular typing,** including the sequencing of seven loci (URA5, CAP59, GPD1, LAC1, PLB1, SOD1, IGS1)

When combined, these **represent the minimum number of genes giving the maximum discriminatory power**

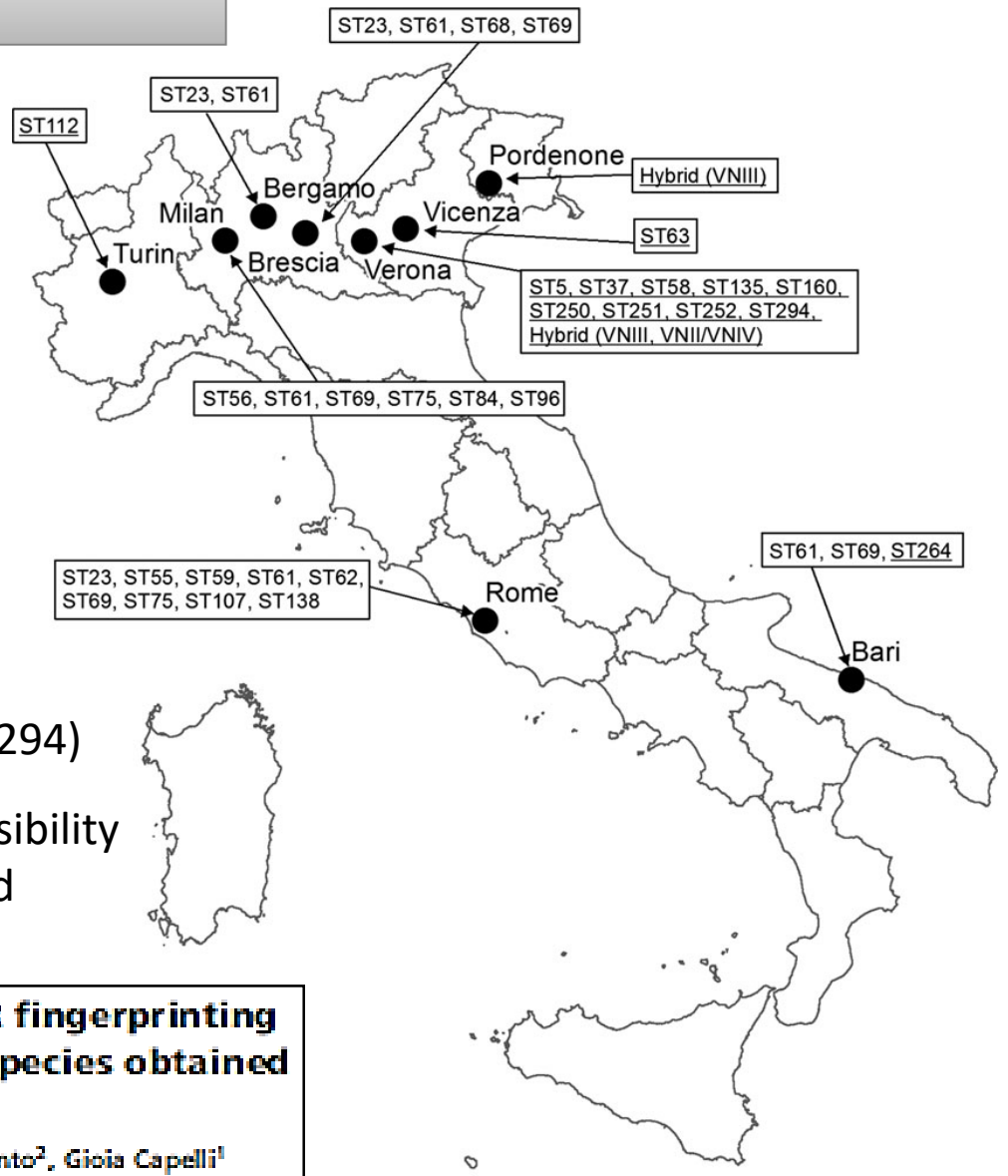
The system assigns

- a progressive sequence code to new sequence

Sequence type	CAP59 Allele	GPD1 Allele	IGS1 Allele	LAC1 Allele	PLB1 Allele	SOD1 Allele	URA5 Allele	Molecular type
Sequence type # 1	7	1	1	1	1	1	1	VNI
Sequence type # 103	7	1	1	2	1	3	2	VNI
Sequence type # 104	7	1	41	18	1	1	1	VNI
Sequence type # 105	7	5	1	3	3	26	1	VNI
Sequence type # 13	1	5	1	1	4	1	1	VNI
Sequence type # 137	1	3	1	4	2	1	5	VNI
Sequence type # 138	7	3	1	2	1	1	1	VNI

Molecular Mycology Research Laboratory - University of Sydney, Sydney, Australia, http://www.mycologylab.org/

Asymptomatic carriage of animals as environmental collector



- High ST variability (12/14)
- New ST (ST 250, ST 251, ST 252, ST 264, ST 294)
- Occurrence of αAD**a** and **a**ADα hybrids: possibility of genetic recombination in the area studied

Multilocus sequence typing (MLST) and M13 PCR fingerprinting revealed heterogeneity amongst *Cryptococcus* species obtained from Italian veterinary isolates

Patrizia Danesi[1,2], Carolina Firacative[3,4], Massimo Cogliati[5], Domenico Otranto[2], Gioia Capelli[1] & Wieland Meyer[3]

Serologic testing and interpretation

- Latex agglutination assay – detection of polysaccharide capsular antigen
- The ALPHA Cryptococcal Antigen enzyme immunoassay (CrAg EIA) - in serum and cerebrospinal fluid (CSF)
- In humans commercial kit- 90% sensitivity and specificity – similarly in pets
- False negative more common in animals with localized ocular, CNS or solitary cutaneous lesions
- Low positive titres may represent subclinical infection
- Antigen titres are frequently extremely high (>1:65,536) in cats and dogs with cryptococcosis
- Good prognosis is support by a decrease in antigen titers over time – 1 step each month

Serologic testing and interpretation

ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2019, 0, 1–8
doi: 10.1093/mmy/myz010
Advance Access Publication Date: 0 2019
Original Article

Original Article

Comparing immunochromatography with latex antigen agglutination testing for the diagnosis of cryptococcosis in cats, dogs and koalas

Mark B. Krockenberger[1,*], Caroline Marschner[1], Patricia Martin[1], George Reppas[2], Catriona Halliday[3], Laura J. Schmertmann[1], Andrea M. Harvey[4] and Richard Malik[5]

Table 1. Sensitivity, specificity, positive predictive value, and negative predictive value for the IMMY CrAg® lateral flow assay in cats, dogs, and koalas when compared to latex cryptococcal antigen agglutination testing.

Species	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Cats	92% (47/51)	81% (63/78)	76% (47/62)	94% (63/67)
Dogs	100% (14/14)	84% (79/94)	48% (14/29)	100% (79/79)
Koalas	98% (131/133)	62% (98/158)	69% (131/191)	98% (98/100)

- IMMY LFA as a screening test to exclude cryptococcosis in diagnostic investigation of **cats, dogs and koalas.**
- A positive LFA result in these species should be confirmed by LCAT testing and/or by aspirate cytology, histopathology, or fungal culture.
- The format of the LFA is suitable for veterinary practice, especially in large busy hospitals and referral centers, as a rapid cage-side POC test to exclude cryptococcosis from the
- differential diagnosis before embarking on other expensive or invasive procedures such as cross-sectional imaging
- In koalas, the test is also suitable for excluding clinical or subclinical cryptococcosis,
- but confirmatory testing of all positive results using the LCAT is again recommended.

Animals as sentinels for human exposure

• Many information about environmental contamination are coming from asymptomatic animals, who, share with human the same environment, but with different behaviour and different susceptibility to ethiological agent

• Exposure to the same highly contaminated environmental source has certainly been associated with outbreaks of disease in groups of animals and humans

• Animals tend to travel much less than poeple

Thanks for your attention